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Abstract: Summary: Stress-related disorders such as PTSD and depression are more prevalent in women than men. One reason for such discordance may be that brain regions involved in stress responses are more sensitive to stress in females. Here, we compared the effects of acute stress on gene transcription in the hippocampus of female and male mice, and also examined the involvement of two key stress-related hormones, corticosterone and corticotropin releasing hormone (Crh). Using quantitative reverse transcription polymerase chain reaction (RT-qPCR), we measured gene expression of Fos, Per1 and Sgk1 45 min after exposure to brief cold swim stress. Stress induced a stronger increase in Fos and Per1 expression in females than males. The handling control procedure increased Fos in both sexes, but occluded the effects of stress in males. Further, handling increased Per1 only in males. Sgk1 was insensitive to handling, and increased in response to stress similarly in males and females. The transcriptional changes observed after swim stress were not mimicked by corticosterone injections, and the stress-induced increase in Fos, Per1 and Sgk1 could neither be prevented by pharmacologically blocking glucocorticoid receptor (GR) nor by blocking Crh receptor 1 (Crhr1) before stress exposure. Finally, we demonstrate that the effects are stressor-specific, as the expression of target genes could not be increased by brief restraint stress in either sex. In summary, we find strong effects of acute swim stress on hippocampal gene expression, complex interactions between handling and sex, and a remarkably unique response pattern for each gene. Overall, females respond to a cold swim challenge with stronger hippocampal gene transcription than males, independent of two classic mediators of the stress response, corticosterone and Crh. These findings may have important implications for understanding the higher vulnerability of women to certain stress-related neuropsychiatric diseases.

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Hippocampal gene expression induced by cold swim stress depends on sex and handling

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Abstract

Stress-related disorders such as PTSD and depression are more prevalent in women than men. One reason for such discordance may be that brain regions involved in stress responses are more sensitive to stress in females. Here, we compared the effects of acute stress on gene transcription in the hippocampus of female and male mice, and also examined the involvement of two key stress-related hormones, corticosterone and corticotropin releasing hormone (Crh). Using quantitative reverse transcription polymerase chain reaction (RT-qPCR), we measured gene expression of *Fos*, *Per1* and *Sgk1* following 45 minutes after brief cold swim stress. Stress induced a stronger increase in *Fos* and *Per1* expression in females than males. The handling control procedure increased *Fos* in both sexes, but occluded the effects of stress in males. Further, handling increased *Per1* only in males. *Sgk1* was insensitive to handling, and increased in response to stress similarly in males and females. The transcriptional changes observed after swim stress were not mimicked by corticosterone injections, and the stress-induced increase in *Fos*, *Per1* and *Sgk1* could neither be prevented by pharmacologically blocking glucocorticoid receptor (GR) nor by blocking Crh receptor 1 (Crhr1) before stress exposure. Finally, we demonstrate that the effects are stressor-specific, as the expression of target genes could not be increased by brief restraint stress in either sex.

In summary, we find strong effects of acute swim stress on hippocampal gene expression, complex interactions between handling and sex, and a remarkably unique response pattern for each gene. Overall, females respond to a cold swim challenge with stronger hippocampal gene transcription than males, independent of two classic mediators of the stress response, corticosterone and Crh. These findings may have important implications for understanding the higher vulnerability of women to certain stress-related neuropsychiatric diseases.

Keywords: stress, sex, hippocampus, corticosterone, Sgk1, Per1

1 Introduction

Women are at a higher risk of developing stress-related affective disorders such as anxiety disorders, post-traumatic stress disorder and major depression. Prevalence of these disorders is estimated to be 1.5-2-fold higher in women than in men (Breslau, 2009; Parker and Brotchie, 2010; Tolin and Foa, 2006). This pronounced sex difference is, at least in part, attributed to heightened stress sensitivity in females (Bangasser and Valentino, 2012; Parker and Brotchie, 2010). While the molecular underpinnings of this difference remain poorly understood, it is clear that the neurobiological effects of stress on brain function depend on the ability of stressors to induce tissue-specific changes in gene expression. The hippocampus is highly sensitive to stress and plays a critical role in the negative effects of stress on cognitive function and on affective disorders (de Kloet et al., 2005). A few studies have demonstrated that the transcriptional response of the immediate early gene *Fos* is more pronounced after acute stress in females than males (Aloisi et al., 1997; Babb et al., 2013). However, it remains unknown whether hippocampal gene expression is generally more responsive to acute stress challenges in females than males, or if this effect is restricted to *Fos*. Further, it is not clear what mechanism is responsible for the increased stress-responsiveness of females. Female rodents show a stronger increase in ACTH and corticosteroid levels than males in response to acute stressors (Goel and Bale, 2010, 2008; Handa et al., 1994), and regulation of corticosteroid receptors in response to acute stress is different in male and female rats (Karandrea et al., 2002). Corticosterone, which acts as a potent regulator of gene expression in the CNS (Datson et al., 2011; Gray et al., 2013), is therefore a likely candidate to mediate some of the sex differences in stress-induced gene expression. Another key regulator of gene expression following stress is corticotropin-releasing hormone (Crh) (Joëls and Baram, 2009). When acting through Crh receptor 1 (Crhr1), it mediates several of the rapid effects of acute stress in the hippocampus (Wang et al., 2013). Sex-differences in stress-induced gene regulation

may therefore be due to differential responsiveness of the Crh system between sexes (Bangasser et al., 2010; Iwasaki-Sekino et al., 2009).

We tested the hypothesis that acute cold swim stress differentially affects the expression of several stress-related genes in male and female hippocampus and investigated whether corticosterone or Crh contribute to this effect. Cold swim stress was chosen as it represents a severe yet brief form of traumatic psychophysiological stress. To this end, we tested the effects of acute cold swim stress on the expression of three well-characterized stress-responsive genes: the activity-dependent immediate early gene *Fos*, and the two corticosterone-sensitive genes Period 1 (*Per1*), a key player in regulating circadian rhythm that is increased in several brain regions in response to acute stress (Takahashi et al., 2001), and serum- and glucocorticoid-inducible kinase 1 (*Sgk1*), a highly conserved stress-responsive protein kinase that regulates a wide variety of cellular processes including neuroexcitability and apoptosis (Lang and Shumilina, 2013).

We demonstrate pronounced swim stress-induced increases in gene expression, which differ markedly between males and females. Effects induced by cold swim stress are stronger in females, and are sensitive to handling particularly in males. We also find that increased transcription of the selected candidate genes in response to acute stress is independent of corticosterone and Crhr1.

2 METHODS

2.1 Animals

C57Bl/6J female and male mice (2.5 months) were obtained from Janvier (France) and maintained in a temperature- and humidity-controlled facility on a 12-hour reversed light–dark cycle with food and water *ad libitum.* These mice were bred in-house to generate animals for the current experiments. After weaning, mice were

housed in groups of 4-5 mice per cage and used for experiments between 2.5 to 6 months of age. For each experiment, mice of the same age were used. 177 mice were used for the current experiments. For initial experiments, larger groups of mice were used, but as the effect size became apparent, we reduced group size in subsequent experiments to reduce the number of mice used in accordance with 3R guidelines. All procedures were carried out in accordance to Swiss cantonal regulations for animal experimentation and were approved under license number 175/2013.

2.2 Stress

For cold swim stress, mice were placed in a plastic cylinder (18 cm high, 13 cm diameter) filled with 18 ± 1°C water up to 12 cm height for 6 min. For restraint stress, mice were placed for 6 min in a 50 ml Falcon tube in which the tip was capped to allow breathing. For control handling, mice were picked up by the base of the tail for about 4 seconds, to mimic the handling involved in the stress procedure. Depending on the experiment, mice were single-housed immediately or 24-hrs before stress/handling. Immediately after stress/handling, mice were returned to their assigned single-housing homecage, and 45 min after initiation of stress/handling, mice were sacrificed by rapid cervical dislocation.

2.3 Drugs

Corticosterone (Sigma) was dissolved in ethanol and diluted into 0.9% saline (5% EtOH) immediately before injection (5mg/kg). Mifepristone (Sigma) and antalarmin (Sigma) were dissolved in DMSO and diluted into 0.9% saline (20% DMSO) immediately before injection (20mg/kg). Dose and route of drug administration was based on previous studies (Furukawa-Hibi et al., 2012; Llorens-Martín and Trejo, 2011; Rissman et al., 2012; Webster et al., 1996). Vehicle injections were either 0.9% saline containing 5% EtOH, or saline containing 20% DMSO. All injections were administered i.p., injection volume was 10ul per gram body mass.

2.4 Tissue collection and processing

Brain tissue. Immediately after sacrifice, the brain was removed and the hippocampus rapidly dissected on ice and stored at -80°C until further processing. RNA was extracted using Trizol (Invitrogen) according to manufacturer's recommendations and on-column DNase treated using the DNase-free RNA kit (Zymo Research). Then, cDNA libraries were generated using Superscript III (Invitrogen), following manufacturer's recommendations, and stored at -20°C until further analyses.

Blood. At the time of sacrifice, trunk blood was collected, stored on ice overnight, then centrifuged at 2000 x g for 10min to collect serum, which was then immediately frozen until further analyses.

Vaginal cytology. For experiment 3, vaginal smears were collected *post mortem* using methods previously described (McLean et al., 2012; Rodgers et al., 2010). Briefly, vaginal cells were obtained by flushing the vaginal opening with 100ul of sterile water, the collected fluid was placed on a glass slide and stained with 0.1% crystal violet for 1min, then washed twice for 1 min with ddH_2O. After drying, smears were analyzed under a light microscope and estrous cycle stage was determined based on the presence/ratio of leukocytes, cornified epithelial cells or nucleated epithelial cells.

2.5 Reverse transcription quantitative real-time PCR (RT-qPCR)

RT-qPCR was performed using SYBR green (Roche) on a Light-Cycler II 480 (Roche) according to manufacturer's recommendations and normalized against Tubulin delta 1 (Tubd1), Hprt hypoxanthine guanine phosphoribosyl transferase (Hprt), and TATA-binding protein (Tbp). Cycling conditions were 5 min at 95°C, then 45 cycles with denaturation (10 sec at 95°C), annealing (10 sec at 60°C), and elongation (10 sec at 72°C). Primers were designed using Primer3Plus (Untergasser et al., 2007) or Quantprime (Wolffe and Matzke, 1999) and tested for quality and specificity by melt-curve analysis, gel electrophoresis and appropriate negative

controls. Forward primer (FP) and reverse primer (RP) sequences were as follows:

Fos: FP: ACAGATACACTCCAAGCGGAGAC, RP: TGGCAATCTCAGTCTGCAACGC

Hprt: FP: GTTGGGCTTACCTCACTGCTTTC, RP: CCTGGTTCATCATCGCTAATCACG

Per1: FP: GAGGGATTTTGGCAGATGAA, RP: GGGACAAGGGGGTTTATTGT

Sgk1: FP: GCCGGTGCCACCCTGGATCTAT, RP: AGGTGCTTGGAGTTCAGGAGCAA

Tbp: FP: TCTGAGAGCTCTGGAATTGTACCG, RP: TGATGACTGCAGCAAATCGCTTG.

Tubd1: FP: TCTCTTGCTAACTTGGTGGTCCTC, RP: GCTGGGTCTTTAAATCCCTCTACG

2.6 Corticosterone ELISA

Serum glucocorticoid levels were measured using a corticosterone ELISA kit (AssayPro, EC3001) following manufacturer's recommendations. Serum samples were diluted 1:100 prior to analysis, and data were analyzed by sigmoidal 4-parameter curve fit using Prism 6 (Graphpad). The minimal detectable dose for this assay is around 40 pg/ml, and intra- and inter-assay coefficients of variation are around 5% and 7%, respectively.

2.7 Statistics

Experiments in males and females were conducted on different days to avoid confounding effects of olfactory cues between sexes. Therefore, RT-qPCR gene expression values were always computed relative to the appropriate same-sex control groups (handling/homecage controls). For comparison of two groups, independent samples t-tests were used. For comparison of >2 groups, one-way ANOVAs were employed if there was a single factor. For two-factorial design (sex x treatment), two-way ANOVAs were employed. Significant effects were further analyzed using LSD post-hoc tests. Statistical significance was set to $p<0.05$. For RT-qPCR, values were *a priori* considered outliers and excluded if they deviated > 2 SDs from the group mean, and/or if melt-curve analysis indicated a poor melt-profile for a given sample.

3 Results

3.1 Experiment 1

To assess the effects of cold swim stress in males and females, mice from a given cage were single-housed immediately before initiation of the experiment. They were then exposed either to cold swim stress or brief handling and tissue was collected 45 min later.

Fos. 2x2 ANOVA showed a significant effect of sex ($F(1,38)=9.06$, $p=0.005$), stress ($F(1,38)=18.66$, $p=0.0001$) and a significant interaction ($F1,38)=7.93$, $p=0.008$, Figure 1A). Post-hoc tests revealed that *Fos* expression after stress was higher in females than in males ($p=0.0001$) and was increased by acute stress in females ($p<0.0001$), but not in males ($p>0.1$).

Per1. 2x2 ANOVA showed a significant effect of sex ($F(1,37)=11.81$, $p=0.002$), stress ($F(1,37)=38.57$, $p<0.0001$) and a significant interaction ($F1,37)=11.81$, $p=0.002$, Figure 1B). Post-hoc tests revealed that *Per1* expression was higher in females than in males ($p<0.0001$) and was increased by acute stress in females ($p<0.0001$) with a trend towards an increase in males ($p=0.078$).

Sgk1. 2x2 ANOVA showed a significant effect of stress ($F(1,38)=17.75$, $p=0.0001$, Figure 1C), but no significant effect of sex and no interaction. Post-hoc tests revealed that *Sgk1* expression was similarly increased by acute stress in both females ($p=0.002$) and males ($p=0.009$).

Corticosterone. 2x2 ANOVA showed a significant effect of sex ($F(1,13)=23.18$, $p=0.0003$) and stress ($F(1,12)=19.19$, $p=0.0007$, Figure 1D) and no significant interaction. Post-hoc tests revealed that corticosterone levels were higher in females compared to males at baseline ($p=0.013$) and following swim stress ($p=0.002$). Corticosterone levels were increased by stress in both males ($p=0.017$) and females ($p=0.004$), Figure 1D).

3.2 Experiment 2

Overall, the findings in experiment 1 suggest a stronger transcriptional response to acute stress in females than males. Surprisingly, we did not observe the expected increase in *Fos* regulation following acute swim stress in males, but only in females. We hypothesized that this may be due to effects of handling and/or single-housing after handling/stress on *Fos* expression. We therefore repeated experiment 1, but this time all mice were single-housed 24hrs before testing. Thus, we avoided that after stress/handling, mice were placed in a novel cage but were returned to their single-housing homecage for 45 mins until they were sacrificed. In addition to the control handling and cold swim stress groups, we also included a homecage control group, which was sacrificed after 24 hrs of single housing without handling or swim stress.

Fos. 2x3 ANOVA (sex x treatment) showed a significant effect of sex ($F(1,23)=13.10$, $p=0.001$), treatment ($F(2,23)=54.11$, $p<0.0001$), and a significant interaction ($F(2,23)=6.54$, $p=0.006$, Figure 1E). In support of our hypothesis, post-hoc tests revealed that handling induced a significant increase in *Fos* expression in both males ($p=0.008$) and females ($p=0.0007$) compared to homecage controls. However, in agreement with our observations from experiment 1, cold swim stress caused an additional increase in *Fos* expression above levels of handling controls only in females ($p<0.0001$), and stress-induced *Fos* levels were much higher in females than in males ($p<0.0001$).

Per1. 2x3 ANOVA showed a significant effect of treatment ($F(2,23)=74.11$, $p<0.0001$), and a significant interaction ($F(2,23)=16.16$, $p<0.0001$, Figure 1F). Follow-up post-hoc tests revealed that handling led to a significant increase in *Per1* expression compared to homecage controls in males ($p=0.007$) but not in females ($p>0.1$). Acute swim stress, however, significantly increased *Per1* expression in both males ($p<0.0001$) and females ($p<0.0001$). In agreement with Experiment 1, the stress-induced increase was greater in females than in males ($p<0.0001$).

Sgk1. 2x3 ANOVA showed a significant effect of treatment ($F(2,23)=17.88$, $p<0.0001$, Figure 1G), and no effect of sex and no interaction. Follow-up post-hoc tests revealed that in males swim stress led to a significant increase in *Sgk1* expression compared to homecage ($p=0.0009$) and handling controls ($p=0.0008$). Similarly, swim stress in females also caused a significant increase in *Sgk1* expression compared to homecage ($p=0.003$) and handling controls ($p=0.002$).

Corticosterone. 2x3 ANOVA (sex x treatment) showed a significant effect of sex ($F(1,23)=34.08$, $p<0.0001$), treatment ($F(2,23)=48.98$, $p<0.0001$), and a significant interaction ($F(2,23)=7.15$, $p=0.004$, Figure 1H). Post-hoc tests reveal that handling slightly increased corticosterone levels only in females ($p=0.015$) but not in males ($p>0.1$). Swim stress increased corticosterone levels in both males ($p=0.0005$) and females ($p<0.0001$) relative to homecage controls. Stress-induced corticosterone levels were higher in females than in males ($p<0.0001$).

3.3 Experiment 3

To assess if some of the observed sex differences may be due to baseline differences in gene expression between males and females, we compared gene expression in male and female mice sacrificed immediately upon removal from their homecage, without any prior stress exposure. No difference in the expression of *Fos*, *Per1* or *Sgk1* was detected between sexes (see Figure 2), indicating that different baseline gene expression in males and females does not account for the different gene expression pattern following handling and/or swim stress.

Because naturally cycling females were used for the current experiments, we also assessed whether the observed sex differences in gene expression after acute stress may be due to fluctuations in baseline gene expression across the estrous cycle. We therefore single-housed female mice for 24 hrs, then sacrificed them for brain extraction and at the same time collected vaginal smears for cycle staging. Of 12 females used, the cycle stage of 10 mice could be unequivocally determined, representative images are shown in Figure 2C. One-way ANOVAs did not reveal any

differences in gene expression for either *Fos*, *Per1* or *Sgk1* between different stages of the cycle (Figure 2B), suggesting that fluctuations in ovarian hormones across the estrous cycle do not seem to regulate target gene expression in females.

3.4 Experiment 4

To examine the mechanism responsible for the rapid increase in gene expression, we hypothesized that corticosterone, the principal stress hormone, may mediate the rapid effects of swim stress in the hippocampus. It is known that corticosterone has widespread effects on gene regulation, both with rapid as well as with delayed onset after stress (Gray et al., 2013; Groeneweg et al., 2012). We thus tested whether an acute injection of corticosterone could mimic the effects of stress on gene expression. Because males have lower corticosterone levels after handling than females (Figure 1H), we used male mice for this experiment. After single-housing for 24 hrs, the animals received an intraperitoneal injection of corticosterone (Sigma, 5mg/kg diluted in 5% EtOH) or vehicle and were sacrificed 45 min later. Corticosterone injection raised serum corticosterone levels significantly (vehicle: 226 ± 41.4 ng/ul; corticosterone: 1277 ± 305.2; $t(13)=3.18$, $p=0.007$). However, as shown in Figure 3 (A-C), corticosterone injection did not increase *Fos*, *Per1* and *Sgk1* levels in the hippocampus, suggesting that the effects observed in response to swim stress are independent of corticosterone.

To more conclusively rule out a role for corticosterone in the rapid effect of stress on gene expression, we used a glucocorticoid receptor (GR) antagonist, mifepristone, to test whether blocking GR can prevent the swim-stress induced increase in gene expression. Females were used for this experiment because they show stronger swim stress-induced effect. They were single-housed for 24 hrs and then received an intraperitoneal injection of mifepristone (20mg/kg, in 5% EtOH) one hour before exposure to swim stress. As before, mice were sacrificed 45 mins after initiation of swim stress. As shown in Figure 3 (D-F), swim stress again led to significant increases in *Fos* ($F(2,13)=16.97$, $p<0.001$), *Per1* ($F(2,13)=25.93$, $p<0.0001$), and

Sgk1 ($F(2,11)=10.16$, $p=0.003$). However, mifepristone did not block the swim-induced increase in gene expression for any of the three genes measured, suggesting that neither the increase in corticosterone levels nor GR-mediated signaling is responsible for the increase in gene expression observed 45min after cold swim stress.

3.5 Experiment 5

Since the rapid increase in gene expression was not mediated by corticosterone, we hypothesized that Crh, a key peptide hormone involved in the stress response and directly released within the hippocampus (Joëls and Baram, 2009), may be necessary for the observed stress-induced changes in gene expression. We therefore tested whether blocking Crh receptor 1 (Crhr1) could prevent the effects of stress on gene expression. Because females show stronger swim stress-induced effects, they were used for this experiment. Mice were single-housed for 24 hrs and then received an intraperitoneal injection of antalarmin (20 mg/kg, in 20% DMSO) one hour before exposure to swim stress. As before, mice were sacrificed 45 mins after initiation of swim stress. ANOVAs demonstrate significant main effects for *Fos* ($F(2,11)=5.08$, $p=0.027$), *Per1* ($F(2,11)=11.52$, $p=0.002$) and *Sgk1* ($F(2,11)=4.39$, $p=0.040$). As shown in Figure 4 (A-C), follow-up post-hoc tests demonstrate that swim stress again increased the expression of *Fos* ($p=0.01$), *Per1* ($p<0.008$), and *Sgk1* ($p=0.046$). However, antalarmin did not block the swim-induced increase in gene expression for any of the three genes measured ($p>0.1$), suggesting that the increase in gene expression observed 45 min after cold swim stress is indepdenent of Crhr1 signaling.

3.6 Experiment 6

Because stress-induced gene expression was independent of corticosterone and Crh, we hypothesized that the observed effects may be stressor specific and may not be induced by other stressors that lead to similar increases in corticosterone and

Crh. To test this hypothesis, we exposed mice either to forced swim stress or restraint stress for the same duration (6 min) and measured *Fos*, *Per1* and *Sgk1* expression relative to a handling control group.

Fos. 2x3 ANOVA (sex x treatment) showed a significant main effect of sex ($F(1,33)=10.81$, $p=0.002$), treatment ($F(2,33)=16.51$, $p<0.0001$) and a significant interaction ($F(2,33)=13.23$, $p<0.0001$, Figure 5A). Post-hoc tests confirmed the results obtained in Experiment 1, showing that swim stress increased *Fos* expression in females ($p<0.0001$) but not in males. Restraint stress, however, failed to increase *Fos* levels compared to handling controls in both males and females.

Per1. 2x3 ANOVA (sex x treatment) showed a significant main effect of sex ($F(1,31)=16.51$, $p=0.0003$), treatment ($F(2,31)=45.96$, $p<0.0001$) and a significant interaction ($F(2,31)=18.08$, $p<0.0001$, Figure 5B). Post-hoc tests confirmed the results obtained in Experiment 1 showing that swim stress increased *Per1* expression in males ($p=0.007$) and females ($p<0.0001$), and the increase is stronger in females compared to males ($p<0.0001$). However, restraint stress failed to increase *Per1* levels compared to handling controls in both sexes.

Sgk1. 2x3 ANOVA (sex x treatment) showed a significant effect of treatment ($F(2,34)=17.27$, $p<0.0001$, Figure 5C) but no significant effect of sex and no interaction. Post-hoc tests confirmed the results obtained in Experiment 1 showing that swim stress increased *Sgk1* expression similarly in males ($p=0.0002$) and females ($p=0.002$). However, restraint stress failed to increase *Sgk1* levels compared to handling controls in both sexes.

Together, these results indicate that the stress-induced increase of candidate genes is stressor specific. While we again confirm the swim stress-induced increase for all target genes, as well as the sex-specific effects of *Fos* and *Per1*, we observe that none of the genes respond to brief restraint stress.

4 Discussion

The aim of the present study was to compare hippocampal gene expression in response to acute stress between males and females. We report that *Fos* and *Per1* both respond more strongly to cold swim stress in females than in males. *Sgk1*, in contrast, responds similarly in both sexes. In addition, we find that several seconds of handling are sufficient to increase *Fos* in both males and females, and *Per1* in males but not females. The handling-induced increase in *Fos* expression in males occludes any further stress-induced increase. We also demonstrate that the observed effects of stress are independent of corticosterone and Crh, suggesting that other peptides or neurotransmitters are likely responsible for the rapid increase following acute stress.

Our finding that gene transcription in response to acute swim stress shows a more pronounced increase in females than in males is in line with previous reports (Aloisi et al., 1997; Babb et al., 2013). Data in humans also suggest that women are more susceptible to suffer from neuropsychiatric disorders classically associated with stressful experiences, such as post traumatic stress disorder (Breslau, 2009; Tolin and Foa, 2006) or depression (Parker and Brotchie, 2010). Our results suggest that stress vulnerability of females may be due to higher transcriptional sensitivity to stress in the hippocampus, a brain region centrally involved in the negative regulation of the stress axis and linked to stress-related neuropsychiatric disease. Sex differences in stress sensitivity are often attributed to differences in HPA axis function (Bangasser and Valentino, 2012; Handa et al., 1994). Indeed, our results confirm previous reports showing that females generally have higher resting levels of corticosterone, and have a more pronounced increase in corticosterone secretion in response to stress (Goel and Bale, 2008; Handa et al., 1994). However, we demonstrate here that the rapid changes in gene expression in response to stress are not mediated by corticosterone. Our results are in agreement with a recent

microarray analysis showing that *Fos*, *Per1* and *Sgk1* are increased by acute swim stress in the male mouse hippocampus, but that these changes are not detected after acute corticosterone injections (Gray et al., 2013). In support of a corticosterone-independent process, stress was found to increase *Fos* expression also after adrenalectomy (Melia et al., 1994). We extend these findings by showing that blocking glucocorticoid receptors does not prevent the stress-induced increase of any of the three target genes. Therefore, despite the marked sex difference in corticosterone levels following stress, this increase does not account for the observed effects of gene expression and is thus unlikely to explain the observed sex differences.

Another key regulator of the HPA axis is Crh, which is locally released in the hippocampus where it can exert rapid effects on gene expression via Crhr1 (Joëls and Baram, 2009). Crh secretion is sexually dimorphic (Bangasser and Valentino, 2012; Iwasaki-Sekino et al., 2009), and Crhr1 signaling is more sensitive in females as G-protein coupling is increased and receptor internalization is decreased compared to males (Bangasser et al., 2010). However, we show that gene expression of the selected candidate genes is independent of Crhr1. The fact that neither corticosterone nor Crh seems to mediate the rapid effects of stress on gene transcription in the hippocampus, is in line with our observation that brief restraint stress – which raises levels of corticosterone and Crh (Chen et al., 2004; Goel and Bale, 2010), is not able to increase gene expression of the tested target genes. Noticeably, prolonged restraint stress (30-100 min) reportedly leads to stronger *Fos* increase in males in several brain regions compared to females (Bland et al., 2005; Figueiredo et al., 2002; Zavala et al., 2011). Therefore, it is likely that other peptide hormones or neurotransmitter systems – which respond specifically to the swim stress challenge – are involved in the strong and rapid regulation of *Fos*, *Per1* and *Sgk1*. Possible neurotransmitter systems involved could be norepinephrine and

serotonin, both of which are very strongly increased after cold swim stress (Gotoh et al., 1998; Linthorst et al., 2008), show sex-specific responses to stressful stimuli (Curtis et al., 2006; Pitychoutis et al., 2012), and can stimulate *Fos* expression (Castro et al., 2003; Gubits et al., 1989). In addition, glutamate may also be involved in the regulation of stress-induced gene expression, as NMDA-receptor antagonists have been shown to block the stress-induced increase in *Fos* expression in the hippocampus (Bozas et al., 1997). *Per1* and *Sgk1* are both activity dependent and can be regulated by CREB (Perrotti et al., 2001; Travnickova-Bendova et al., 2002). Thus, glutamate-mediated calcium increase likely triggers rapid gene transcription in response to stressful stimuli.

Additionally, the fact that all three genes assessed in this study show different response patterns between sex, handling and stress, suggests that distinct mechanisms may regulate their expression. It is known that the stress response is highly complex and acts through many parallel signaling networks (Joëls and Baram, 2009), thus it remains to be established which signals control the widespread transcriptional alterations rapidly induced by acute stress throughout many brain regions. Notably, the fact that gene expression is affected only by cold swim stress but not restraint stress may suggest that the severity of the stress or physiological components of the stressor (exhaustion, physical activity, hypothermia) may be responsible for target gene regulation in a stressor-specific manner. It is possible that females are more vulnerable/responsive to severe psychophysiological stressors, a hypothesis that needs to be investigated in future studies.

Per1 is a key regulator of the circadian clock and can respond rapidly to light stimulation (Okamura, 2007), yet relatively little is still known about the involvement of *Per1* in the stress response. Our data confirm previous reports showing that *Per1* can be rapidly regulated by acute stressors in several brain regions (Gray et al., 2013; Yamamoto et al., 2005) and even in peripheral tissues (Yamamoto et al.,

2005). However, we are the first to show that *Per1* expression is extremely sensitive to a very short period of handling in males. While baseline expression of *Per1* is similar between males and females, handling and stress reveal sex dependent effects. Previous work has already demonstrated that handling can interact with the effects of ovarian hormones to impact hippocampus sensitive behaviors (Bohacek and Daniel, 2007), so the effect of ovarian hormones on *Per1* expression needs to be explored in future experiments. Indeed, *Per1* expression in the suprachiasmatic nucleus brain clock has been shown to be sensitive to circulating androgen levels (Karatsoreos et al., 2011), raising the possibility that steroid hormones can have modulatory effects on *Per1* expression in different brain regions, possibly in concert with stress-hormones. Importantly, the present work used naturally cycling females, without controlling for the hormonal fluctuations occurring across the estrous cycle that may affect the responsiveness to stress (Figueiredo et al., 2002). Although we did not observe differences in baseline gene expression levels across different stages of the cycle (Figure 2B), we did not assess the effects of acute swim stress on females at different stages of their cycle. Previous work has indicated that the gene expression in response to acute stress can be sensitive to cycle stage in females (Figueiredo et al., 2002). However, we think it is unlikely that circulating hormone levels had a strong impact on the stress-induced gene expression changes described in the present work. First, across many different experiments described here we always observed only small within-group variation in females, comparable to the variation observed in males, and confounding variables (i.e. cycle stage) would be expected to increase standard deviation. Second, many of the experiments presented here were conducted on different days in various cohorts of mice. This means that in all likelihood females were tested at various stages of their cycle, yet we observed very similar gene expression responses across experiments.

Sgk1 is a known target involved in various forms of cell stress and regulates several fundamental processes including ion channel function and neuronal excitability (Lang and Shumilina, 2013; Pearce, 2003). *Sgk1* has also been proposed to play a critical role in memory performance, as fast learning rats have higher levels of *Sgk1* mRNA than slow learners, and *Sgk1* inhibition impairs, while *Sgk1* overexpression enhances memory consolidation (Lee et al., 2007). The rapid activation of *Sgk1* in response to acute stress could thus be important for the effects of stress on memory performance, as it is well-known that acute stress can enhance memory acquisition (Sandi et al., 1997). Our data confirm a previous report that acute swim stress induces *Sgk1* expression in the hippocampus (Gray et al., 2013). We demonstrate that *Sgk1* expression is regulated similarly in males and females, and is insensitive to the effects of handling. Previous work has demonstrated that even after a prolonged period of severe stress – 6 hrs of restraint water submersion – a similar two-fold increase in *Sgk1* mRNA levels could be detected (Murata et al., 2005), suggesting that a sufficiently strong stressor can cause rapid and persistent activation of *Sgk1* in the hippocampus. Our finding that stress-induced *Sgk1* induction is independent of corticosterone and Crhr1 is in contrast with in vitro studies that report increased *Sgk1* expression by corticosterone in cultured hippocampal progenitor cells (Anacker et al., 2013), as well as by Crh via Crhr1 signaling (Sheng et al., 2008). In vivo studies, however, have confirmed our observation that *Sgk1* was not increased in the hippocampus after chronic nor acute high-dose corticosterone injections in rats and mice (Gray et al., 2013; Neeltje G van Gemert et al., 2006). Notably, however, a substantial increase in *Sgk1* levels was detected one hour after an acute corticosterone injection in the corpus callosum, a region immediately adjacent to the hippocampus, demonstrating a remarkable regional specificity of *Sgk1* regulation (van Gemert et al., 2006). Together with our results this suggests that *Sgk1* may be regulated by corticosterone in several brain regions as well as in *in vitro* systems, but there is complex region-specific regulation of *Sgk1* in vivo and corticosterone alone

does not recapitulate the stress-induced increase of *Sgk1* expression in the hippocampus.

In summary, our data suggest that acute stress engages several different signaling pathways that rapidly orchestrate a gene-specific pattern of transcription in the hippocampus. These effects are sensitive to sex and handling, independent of the classic HPA-axis regulators corticosterone and Crh, and tend to be more pronounced in females, at least in response to cold swim stress. These findings have important methodological implications for studying the effects of stress paradigms in males and females, as they show that handling involved in control procedures can lead to sex-specific effects that can occlude the effects of stress. Future studies will need to elucidate which peptides or neurotransmitter systems mediate these rapid effects of acute stress, and test whether the identified molecular differences can explain behavioral differences between males and females in the responsiveness to acutely stressful situations.

Conflict of interest

The authors confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

Contributors

JB designed and conducted all experiments, collected and processed tissue, analyzed data and wrote the manuscript; FM and MR conducted the experiments, processed tissue and ran qPCR analyses; IM funded the research and wrote the manuscript.

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6 Figure Captions

Figure 1. Effect of acute cold swim stress on hippocampal gene expression and corticosterone levels in males and females. Swim stress increases hippocampal gene expression of *Fos, Per1* and *Sgk1* in mice that are single housed immediately before stress/handling (A-C; males: n=7-10/group; females: n=12/group). Stress also increases target gene expression in mice that are single-housed 24 hours before stress/handling (E-G; males: n=5/group, females: n=4-5/group). Stress also raises blood corticosterone levels in mice that are single-housed immediately before stress/handling (D, males: n=4-5/group; females: n=4/group), and similarly in mice that are single-housed for 24 hours before stress/handling (H; males: n=5/group, females: n=4-5/group). Data represent mean ± SEM. #=p<0.08; *=p<0.05; **=p<0.01; ***=p<0.001; ****=p<0.0001.

Figure 2. Baseline hippocampal gene expression in male and female mice and across the estrous cycle. After 24 hrs of single housing there are no differences between *Fos, Per1* and *Sgk1* expression between males and females (A; n=4-5/group). Gene expression does not change across the different stages of the female estrous cycle (n=3-4/group). Representative images of vaginal smears used for cycle staging are shown (C). Data represent mean ± SEM.

Figure 3. Effect of corticosterone on hippocampal gene expression. I.p. injection of corticosterone (5 mg/kg) 45 min before tissue collection does not alter gene expression of *Fos, Per1* and *Sgk1* in the hippocampus (A-C; vehicle: n=7; corticosterone: n=8). The glucocorticoid receptor antagonist mifepristone (20 mg/kg) injected i.p. one hour before swim stress does not block the stress-induced increase in gene expression (D-F, n=5-6/group). Data represent mean ± SEM. ***=p<0.001; ****=p<0.0001.

Figure 4. Effect of Crhr1 antagonist on stress-induced hippocampal gene expression. I.p. injection of the Crhr1 receptor antagonist antalarmin (20 mg/kg) one hour before swim stress does not block the stress-induced increase in gene expression (D-F; n=4-5/group). Data represent mean ± SEM. *=p<0.05; **=p<0.01.

Figure 5. Effect of brief swim or restraint stress on hippocampal gene expression. Cold swim stress for 6 min increases gene expression of *Fos, Per1* and *Sgk1* in the hippocampus, but the same duration of restraint stress does not increase target gene expression (A-C; males: n=8-10/group; females: n=4-5/group). Data represent mean ± SEM. **=p<0.01; ***=p<0.001; ****=p<0.0001.

Figure 1.

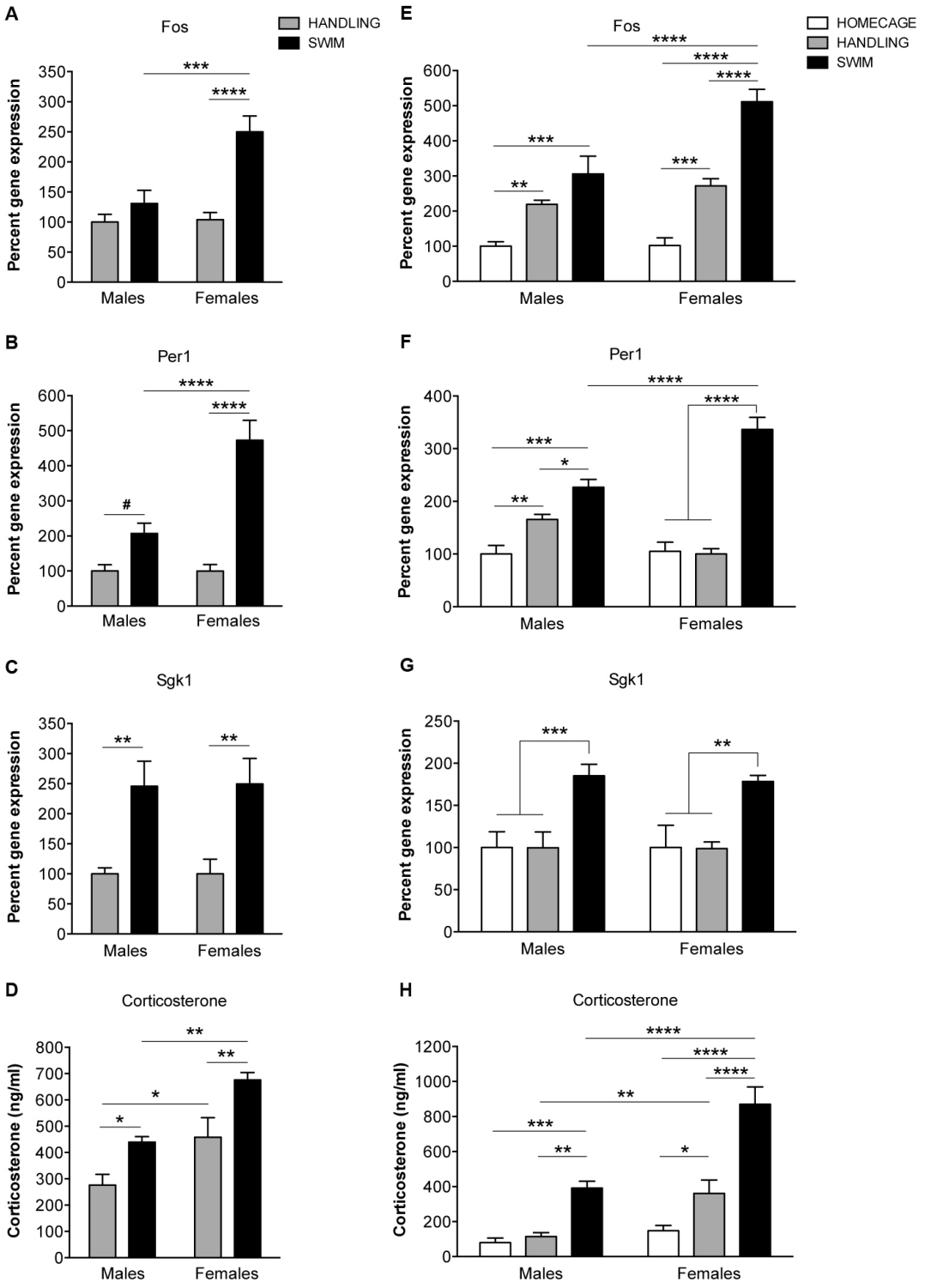

A
Fos
HANDLING
SWIM
Percent gene expression
Males
Females
B
Per1
C
Sgk1
D
Corticosterone
Corticosterone (ng/ml)
E
HOMECAGE
F
G
H

Figure 2.

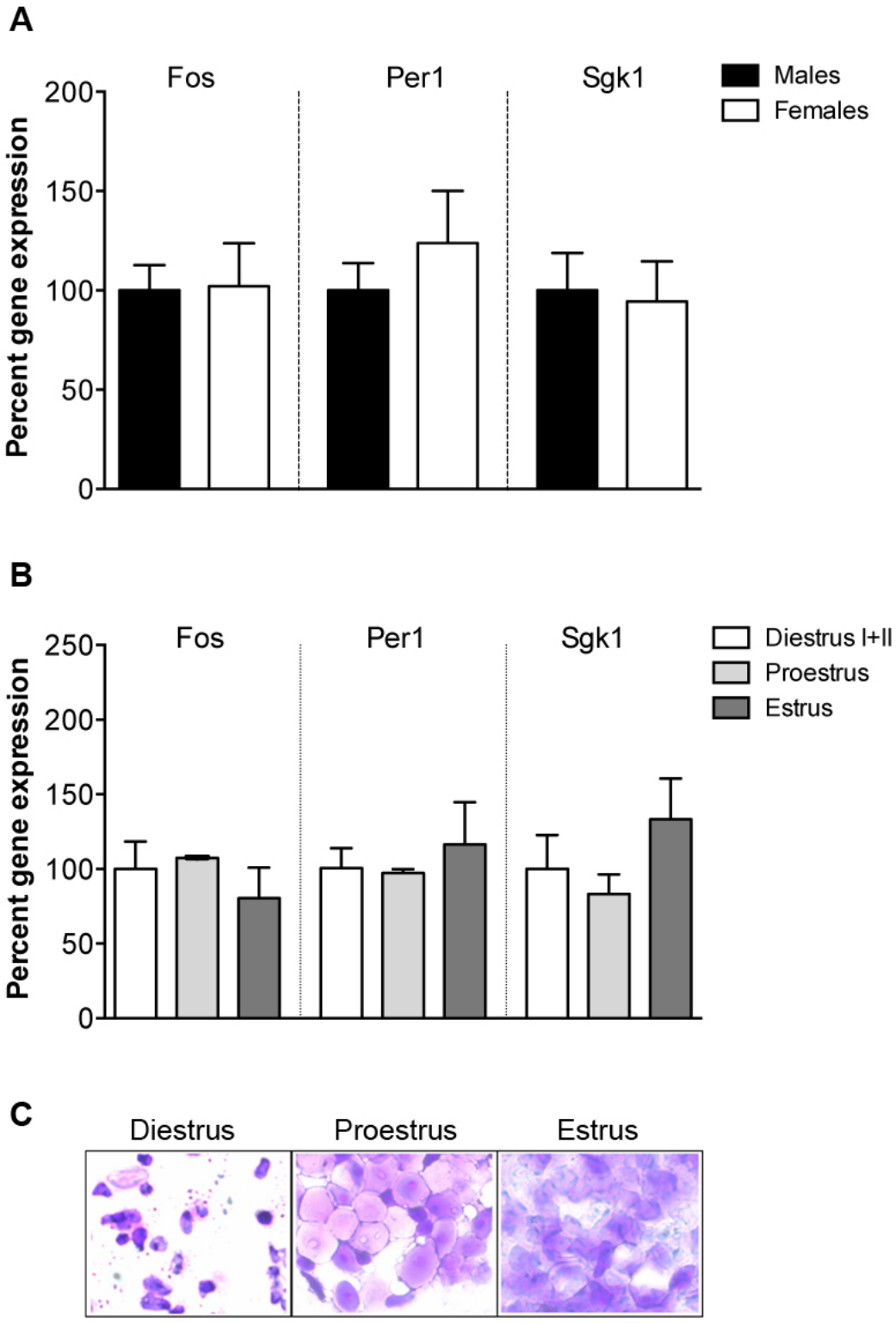

A
Fos
Per1
Sgk1
Males
Females
Percent gene expression
200
150
100
50
0
B
Fos
Per1
Sgk1
Diestrus I+II
Proestrus
Estrus
Percent gene expression
250
200
150
100
50
0
C
Diestrus
Proestrus
Estrus

Figure 3.

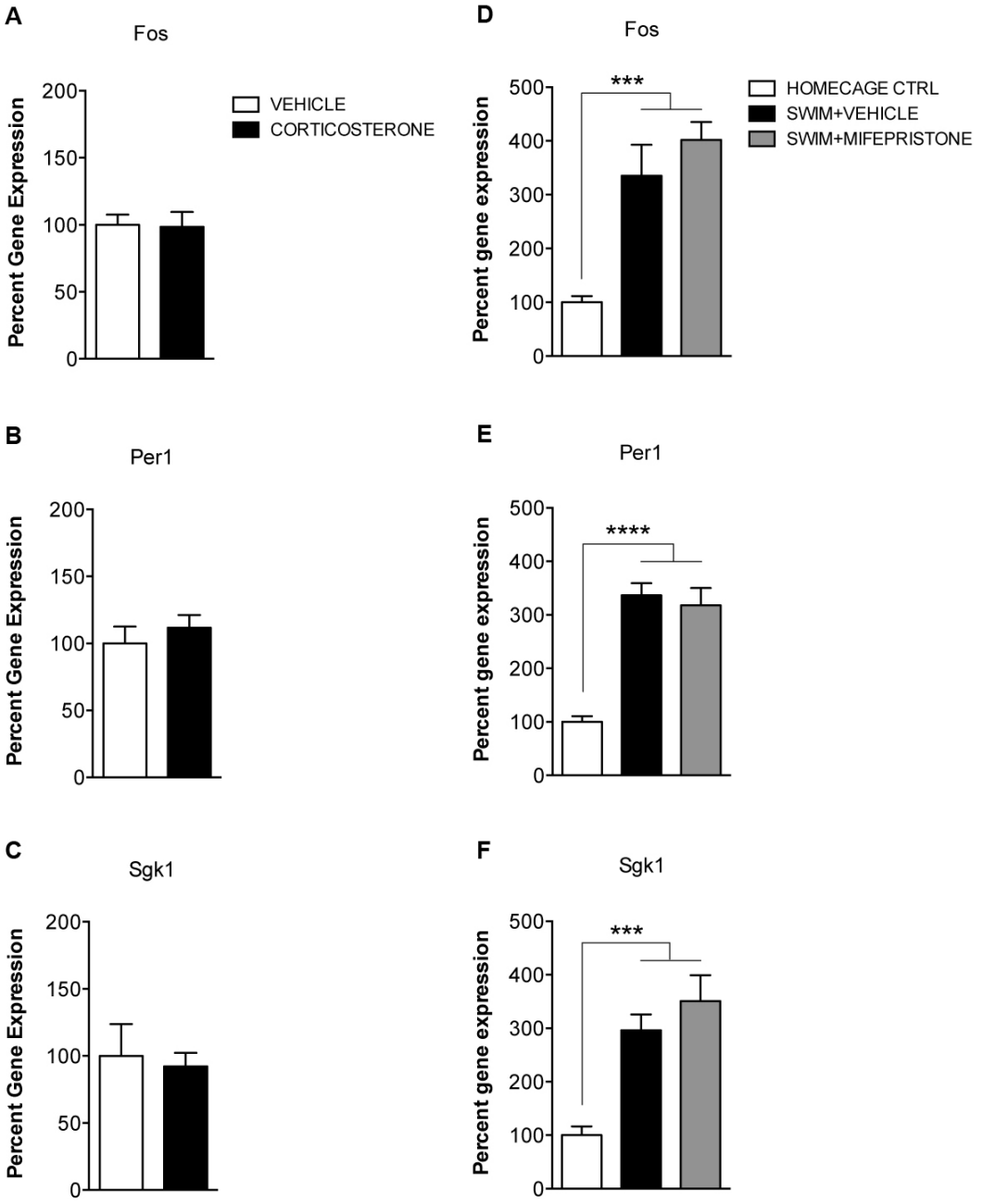
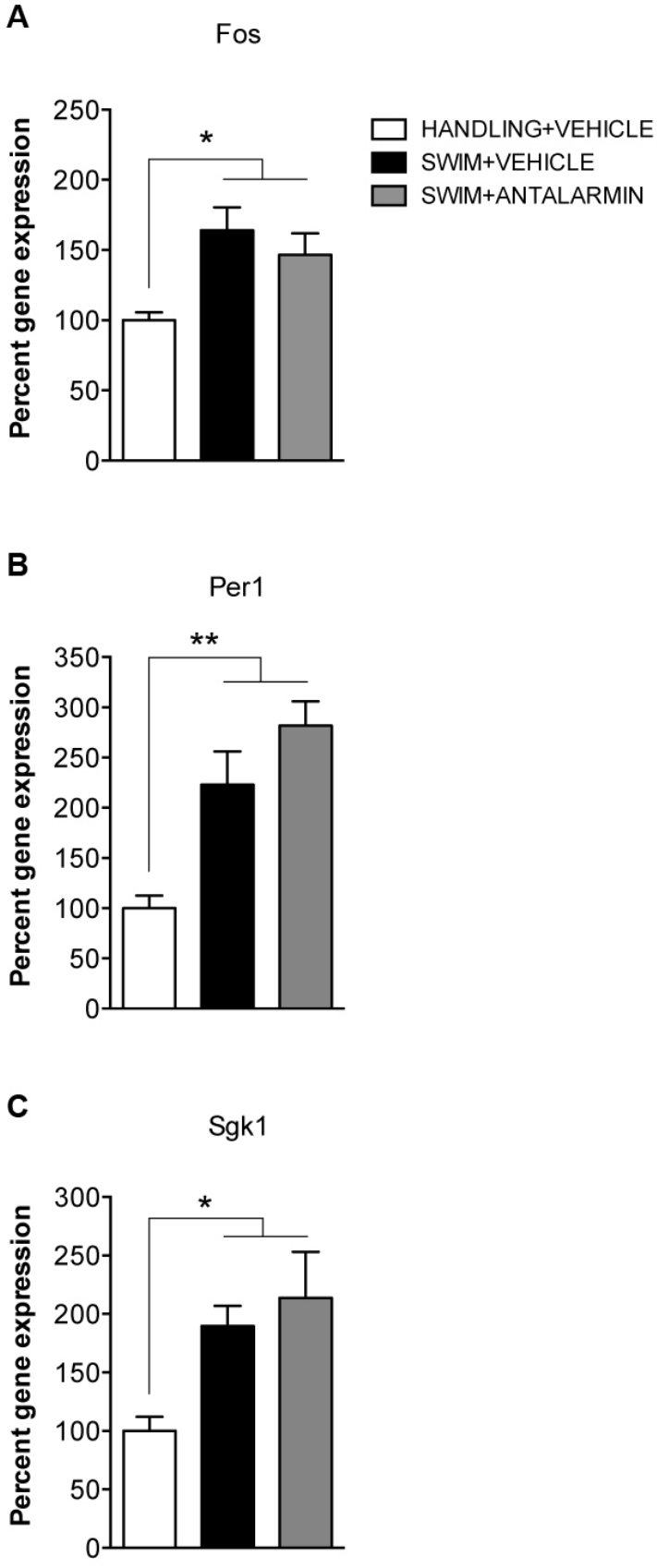

A
Fos
Percent Gene Expression
200
150
100
50
0
VEHICLE
CORTICOSTERONE
B
Per1
Percent Gene Expression
200
150
100
50
0
C
Sgk1
Percent Gene Expression
200
150
100
50
0
D
Fos
***
Percent gene expression
500
400
300
200
100
0
HOMECAGE CTRL
SWIM+VEHICLE
SWIM+MIFEPRISTONE
E
Per1
****
Percent gene expression
500
400
300
200
100
0
F
Sgk1
***
Percent gene expression
500
400
300
200
100
0

Figure 4.


A
Fos
Percent gene expression
250
200
150
100
50
0
*
HANDLING+VEHICLE
SWIM+VEHICLE
SWIM+ANTALARMIN
B
Per1
Percent gene expression
350
300
250
200
150
100
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**
C
Sgk1
Percent gene expression
300
250
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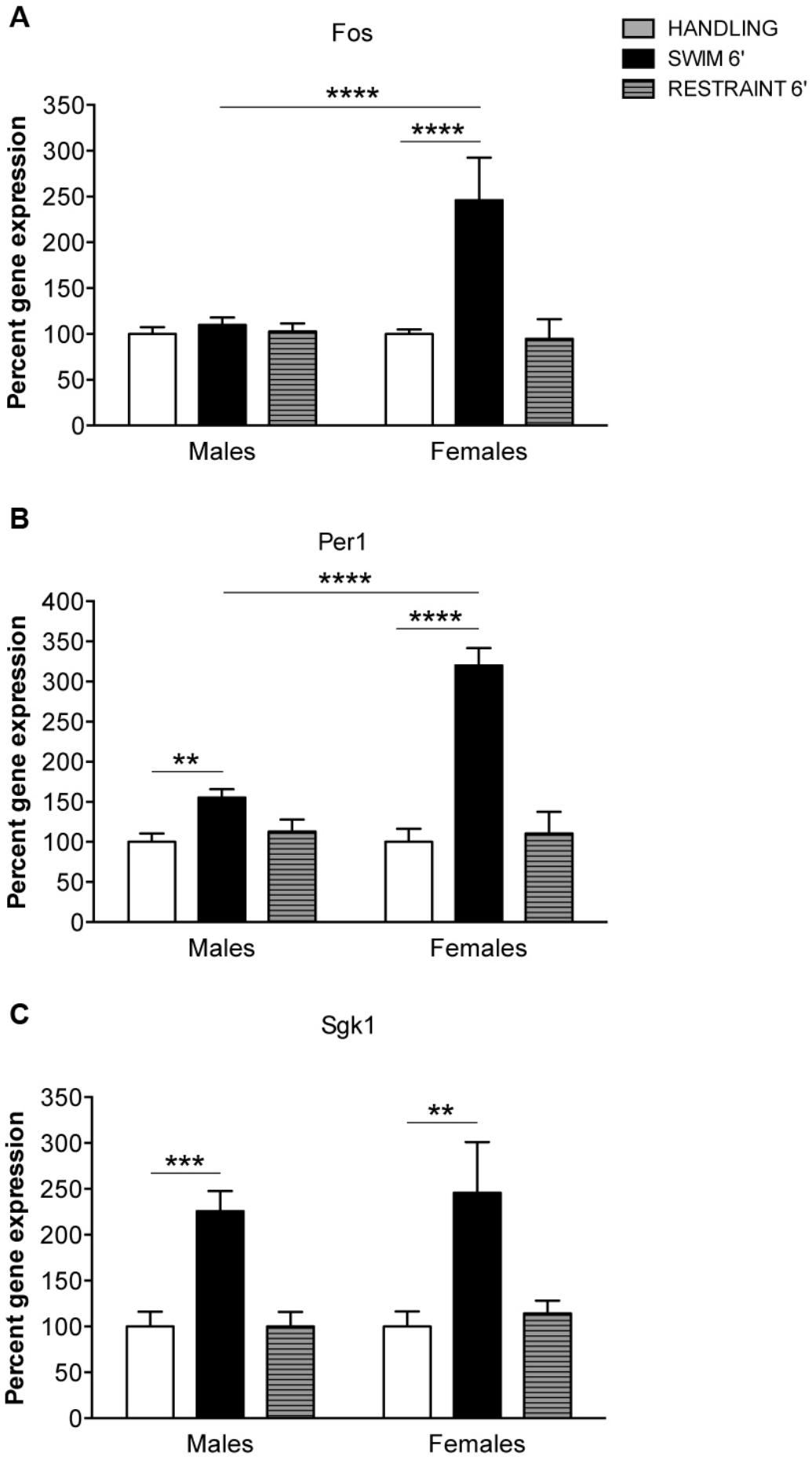
Figure 5.


A
Fos
HANDLING
SWIM 6'
RESTRAINT 6'
****
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Percent gene expression
350
300
250
200
150
100
50
0
Males
Females
B
Per1
****
****
**
Percent gene expression
400
350
300
250
200
150
100
50
0
Males
Females
C
Sgk1
***
**
Percent gene expression
350
300
250
200
150
100
50
0
Males
Females